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Interaction between ArgR and AhrC Controls Regulation of Arginine Metabolism in *Lactococcus lactis**

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The expression of arginine metabolism in *Lactococcus lactis* is controlled by the two homologous transcriptional regulators ArgR and AhrC. Genome sequence analyses have shown that the occurrence of multiple homologues of the ArgR family of transcriptional regulators is a common feature of many low-G + C Gram-positive bacteria. Detailed studies of ArgR type regulators have previously only been carried out in bacteria containing single regulators. Here, we present a first characterization of the two *L. lactis* arginine regulators by means of gel retardation and DNase I footprinting. ArgR of *L. lactis* was shown to bind to the promoter regions of both the arginine biosynthetic *argCJDBF* operon and the arginine catabolic *arcABD1C1C2TD2yvaD* operon, but in an arginine-independent manner. Surprisingly, AhrC alone was unable to bind to DNA. Arginine-dependent DNA binding was obtained by mixing the two regulators in gel retardation assays. With both regulators present, the addition of arginine led to increased binding of ArgR-AhrC to the biosynthetic *argC* promoter but also to diminished binding to the catabolic *arcA* promoter. Footprinting showed ArgR-AhrC protection of regions containing ARG box operator sequences preceding *argC*. In the absence of AhrC, ArgR protected sites in the *arcA* promoter region with similarity to ARG box half-sites, here called ARC boxes. We propose a model for repression of arginine biosynthesis and activation of catabolism by anti-repression, involving arginine-dependent interaction between the two *L. lactis* regulator proteins, ArgR and AhrC.

Despite differences in the organization of genes involved in arginine metabolism, experimental evidence indicates that the mechanism of arginine-dependent regulation of these genes is highly conserved among a range of different organisms, including Gram-negative, Gram-positive and extremophilic bacteria (1–12). Regulation is exerted by binding of single transcriptional regulators of the ArgR family to so-called ARG operator sites preceding the relevant target genes, generally leading to repression of arginine biosynthetic genes and activation of catabolic genes, in the presence of arginine.

Crystal structures of the ArgR type transcriptional regulators of *Escherichia coli* (ArgR*Ec* (13, 14)), *Bacillus stearothermophilus* (ArgR*Bst* (15)), and *Bacillus subtilis* (AhrC*Bsu* (16)) have revealed these to be structurally similar proteins, making up a complex of six identical subunits. The subunits are arranged in hexameric structures, which are organized as dimers of trimers. In *E. coli* and *B. subtilis*, the hexameric structure is maintained both in the absence and presence of arginine (4, 17), whereas the regulator of *B. stearothermophilus* mainly exists as a trimer that assembles into hexamers dependent of the concentrations of arginine, protein, and DNA (5, 15). Six arginine molecules are bound at the trimer-trimer interface, strengthening the interaction between the trimers and at the same time introducing a conformational change in the regulator, thus increasing its affinity for operator binding (4).

An ArgR monomer consists of an N-terminal DNA-binding domain, a central hinge region, and a C-terminal multimerization and arginine-sensing domain. In hexameric form, the DNA-binding domains surround the core of C-terminal domains (14–16). Mutagenesis studies of mainly ArgR*Ec* have allowed identification of specific amino acid residues making up the N-terminal winged helix-turn-helix DNA binding region (18). Additionally, a range of residues in the C-terminal domain has been shown to be important for either subunit multimerization or arginine binding (18–20).

ARG operator sites consist of pairs of 18-bp palindromic sequences (called ARG boxes), of which the 5′-TnTGnATwwwwATnCAnA-3′ (where conserved residues are capitalized, n represents any nucleotide, and w represents A or T) consensus sequence in *E. coli* (21) is conserved with only small variations in various other organisms studied (22). The distance between the ARG boxes varies between 2 bp (*e.g.* for the *B. stearothermophilus argC* operator) and 3 bp (for the *E. coli* biosynthetic $argC_{O1}$ operator). This spacing means that the boxes are aligned on the same side of the DNA helix. Also, single ARG boxes can confer regulator binding and regulation. This is exemplified by the arginine catabolic *rocABC* and *rocDEF* operons of *B. subtilis* (23–25) and the biosynthetic *argGHCJBD* operon of *Thermotoga maritima* (9). ARG box sequence variation, spacing, and location are factors that determine the strength of regulator-DNA interaction.

Whereas single ArgR-type regulators have been studied in detail, the continuously increasing number of bacterial genome sequences becoming available make it clear that several low-G + C Gram-positive organisms harbor multiple homologues of ArgR type regulators (see overview by Belitsky (26)). A few recent investigations have proven that these ArgR homologues are not merely orthologous but fulfill distinct functions in these organisms. A study in *Enterococcus faecalis* revealed the presence, upstream of the arginine catabolic *arcABCRD* operon, of two genes named *argR1* and *argR2* (10). Although the function of the *E. faecalis* ArgR-type regulators was not investigated, it was proven that the divergently transcribed *argR1* and *argR2* genes were differentially expressed in response to arginine and glucose, possibly via putative ARG boxes preceding the genes (10). In our laboratory, a random knock-out screening led to the

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TABLE I
Bacterial strains and plasmids

Name	Characteristics	Source or reference
L. lactis		
MG1363	*L. lactis* ssp. *cremoris*, plasmid-free derivative of NCDO 712	Ref. 41
NZ9000	MG1363Δ*pepN*::*nisRK*	Ref. 42
MGΔ*argR*	MG1363, with unmarked deletion of *argR*	Ref. 12
MGΔ*ahrC*	MG1363, with unmarked deletion of *ahrC*	Ref. 12
MGΔ*argRahrC*	MG1363, with unmarked deletion of *argR* and *ahrC*	Ref. 12
NZΔ*ahrC*	NZ9000, with unmarked deletion of *ahrC*	This work
NZΔ*argRahrC*	NZ9000, with unmarked deletion of *argR* and *ahrC*	This work
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}ZΔ *M15 Tn10* (TetR)]	Stratagene
Plasmids		
pORI280	Em^R *ori*+ *repA*-; deletion derivative of pWV01; constitutive *lacZ* expression from P32	Ref. 43
pVE6007	Cm^R *repA*+; Ts-*ori* derivative of pWV01	Ref. 33
pNG8048E	Cm^R Em^R; Nisin inducible P*nisA*. Em^R-derivative of pNZ8048	Laboratory stock
p280Δ*argR*	Em^R; pORI280 containing *argR* deletion construct	Ref. 12
p280Δ*ahrC*	Em^R; pORI280 containing *ahrC* deletion construct	Ref. 12
pNG-ArgR	Cm^R; wild type ArgR under control of the nisin-inducible *nisA* promoter in pNG8048E	This work
pNG-AhrC	Cm^R; wild type *ahrC* under P*nisA* control in pNG8048E	This work
pNG-HisArgR	Cm^R; N-terminal His-tagged ArgR under P*nisA* control	This work
pNG-HisAhrC	Cm^R; N-terminal His-tagged AhrC under P*nisA* control	This work
pUC19	Amp^R; *E. coli* high copy cloning vector	Ref. 35
pUC-ArgR	Amp^R; wild type *argR* blunt end-cloned in the SmaI site of pUC19	This work
pUC-AhrC	Amp^R; wild type *ahrC* blunt end-cloned in the SmaI site of pUC19	This work
pUC-R126	Amp^R; pUC19 carrying ArgR(A126D) point mutation	This work
pUC-R127	Amp^R; pUC19 carrying ArgR(D127G) point mutation	This work
pUC-C124	Amp^R; pUC19 carrying AhrC(D124G) point mutation	This work
pUC-C126	Amp^R; pUC19 carrying AhrC(D126G) point mutation	This work
pNG-R126	Cm^R; ArgR(A126D) under P*nisA* control in pNG8048E	This work
pNG-R127	Cm^R; ArgR(D127G) under P*nisA* control in pNG8048E	This work
pNG-C124	Cm^R; ArgR(D124G) under P*nisA* control in pNG8048E	This work
pNG-C126	Cm^R; ArgR(D126G) under P*nisA* control in pNG8048E	This work

identification of the *argR* and *ahrC* genes in *L. lactis*, the gene products of which were responsible for repression of the arginine biosynthetic *gltSargE* operon (12). Further characterization showed that both ArgR and AhrC of *L. lactis* are necessary for repression of the arginine biosynthetic *argCJDBF*, *gltSargE*, and *argGH* operons; they do not complement each other. Interestingly, arginine-dependent regulation of the arginine catabolic *arcABD1C1C2TD2yvaD* operon also required both ArgR and AhrC, but in a manner different from that of arginine biosynthesis. Whereas deletion of *argR* resulted in constitutively increased expression of the *arc* genes, deletion of *ahrC* gave constitutively decreased expression. However, *arc* expression was increased in an *L. lactis argR ahrC* double mutant, indicating that AhrC is not a classical activator of *arc* expression and, additionally, that ArgR might act as a repressor of *arc* expression (12). A thorough recent study of arginine regulation in *Lactobacillus plantarum* showed that repression of arginine biosynthesis was abolished when point mutations were introduced in either one of two separate genes encoding putative ArgR-type regulators or in promoter regions containing ARG box-like sequences (11).

In this work, we have sought to clarify the molecular basis for the complex dual mechanism of ArgR-AhrC-mediated regulation in *L. lactis*. To this end, purified ArgR and AhrC were investigated for their function in DNA binding and arginine sensing, with respect to both repression of arginine biosynthesis and activation of catabolism. The experimental evidence allowed us to propose a comprehensive model of ArgR-AhrC-mediated gene regulation in *L. lactis*.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Media—Strains of *Lactococcus lactis* ssp. *lactis* (listed in Table I) were routinely cultivated at 30 °C in M17 (27) medium containing 0.5% (w/v) glucose (GM17). For primer extensions and citrulline determinations, cells were grown in a chemically defined medium (CDM15) as described previously (28), with 0.5% (w/v) glucose as carbon source and free amino acids as nitrogen source. Arginine was added to the CDM15 as described throughout. When required, 4 μg/ml erythromycin (*Em*), 4 μg/ml chloramphenicol (*Cm*), or 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to the growth medium. Chemicals and antibiotics were purchased from Merck and Sigma. For induction of genes cloned behind the *nisA* promoter, Nisaplin (Aplin & Barnett Ltd., Beaminster, Dorset, UK) was suspended 1:1 (w/v) in 50% ethanol, thoroughly vortexed, and centrifuged (5 min at 12,000 rpm), after which the supernatant was added 1:1 × 10^{-6} (v/v) to the culture, unless stated otherwise.

DNA Isolation and Manipulation—General molecular techniques were performed as described by Sambrook *et al.* (29). Chromosomal and plasmid DNA was isolated from *L. lactis* according to Johansen and Kibenich (30) and Birnboim (31), respectively. *L. lactis* and *E. coli* were transformed with plasmid DNA by electroporation as described by Holo and Nes (32) using a Bio-Rad Gene Pulser (Bio-Rad). All DNA modification enzymes were purchased from Roche Applied Science, and used according to the manufacturer's directions. PCRs were performed using *Pwo* DNA polymerase (Roche Applied Science) and purified with the Roche PCR purification kit (Roche Applied Science). Primers (listed in Table II) were purchased from Biolegio BV (Malden, The Netherlands).

Construction of Regulator Deletion Mutants of NZ9000—Since *L. lactis* strains MG1363 and NZ9000 are isogenic, the *argR* and *ahrC* deletion plasmids pORIΔ*argR* and pORIΔ*ahrC*, made with MG1363 chromosomal DNA as template (12), were used to delete these genes from NZ9000. Single crossover integration and excision in NZ9000 was done using pVE6007 (33) as helper plasmid, as described before (12), yielding *L. lactis* NZΔ*ahrC* and *L. lactis* NZΔ*argRahrC* (Table I). Chromosomal deletions were confirmed by PCR and by Southern blotting. Probe-labeling, hybridization, and detection was done with the ECL direct nucleic acid labeling system (Amersham Biosciences), according to the manufacturer's instructions.

Overexpression and Isolation of His-tagged ArgR and AhrC Proteins—The *argR* and *ahrC* genes were amplified from MG1363 chromosomal DNA with the primer pairs argR-Nhis1/argR-MAL2 and ahrC-Nhis1/ahrC-His2, respectively, thereby introducing N-terminal hexahistidine tags (His tags). The PCR products were cloned as NcoI/HindIII and NcoI/XbaI fragments, respectively, in the multiple cloning

TABLE II
Primers used in this study

Name	Sequence (5′ → 3′)
argR-NZ	CGTCATGAAAAGAGATAAAAG
argR-NHis-1	CATGCCATGGGACATCATCATCATCATCATATGAAAAGAGATAAAAGATTAG
argR-MAL2	CCCCAAGCTTAACCTAATAAAATATATT
ahrC-5	CGGAATTCACAATGAATAAGCTGCC
ahrC-NZ	CGTCATGAAAAGAGAAGAACG
ahrC-NHis1	CATGCCATGGGACATCATCATCATCATCATATGAAAAGAGAAGAACGATTAAAC
ahrC-His2	GCTCTAGATTAAAACTCTGTCAG
ahrC-MG(D124G)-1	CAATTATCATTGGTGACGATAGTGCC
ahrC-MG(D124G)-2	GGCACTATCGTCACCAATGATAATTG
ahrC-MG(D126G)-1	CATTGATGACGAGAGTGCCTTGGTTAT
ahrC-MG(D126G)-2	ATAACCAAGGCACTCTCGTCATCAATG
argR-MG(A126D)-1	GGTACAATTGCCGGTGATGATACCTTGCTTG
argR-MG(A126D)-2	CAAGCAAGGTATCATCACCGGCAATTGTACC
argR-MG(D127G)-1	CAATTGCCGGTGCTGAGACCTTGCTTGTTATTTG
argR-MG(D127G)-2	CAAATAACAAGCAAGGTCTCAGCACCGGCAATTG
argC-2	GCTCTAGATATAACCTCTAATTCCG
argC-5	CGGAATTCGTTTAAAAAAGTATAATAATAC
argC-7	CTAAAAAATTAATGAATTATC
arcA-1	CGGAATTCATTCTTGCTGATGAGAG
arcA-3	CGGAATTCAAATATTTTGTAAAATAAG
arcA-4	CGGAATTCGAATCCCATGATAAGC
arcA-5	CGGAATTCAACGTGAAATTGTCAG
arcA-6	CGGAATTCTATAAATGAATAAACC
arcA-7	CGGAATTCAAAATATGCATAGATG
arcA-10	GTTAACTCAGAAATTGGG
arcA-1rev	GCTCTAGACACTCCTTTGCTTATC
arcA-3rev	GCTCTAGAACATGTTTTTTTATATTCTG
arcA-4rev	GCTCTAGAGCACTAATGCTACAC
arcA-5rev	GCTCTAGAATTATACATCTATGC
arcA-6rev	GCTCTAGAGATTCAGTATTATTGG
arcA-7rev	GCTCTAGATTAACATTAATTCCATTG
arcA-10rev	GCTCTAGACAAATAAAAGCTGTTTC
glnA-3	CGCGGATCCTATATTAGTAAAGTTC
glnA-5	AACTGCAGGCACTGGGCGAACACGTTTTGG
arcA-px	CATCAAATAAAAGCTGTTTCATTGTGTCTGGGG

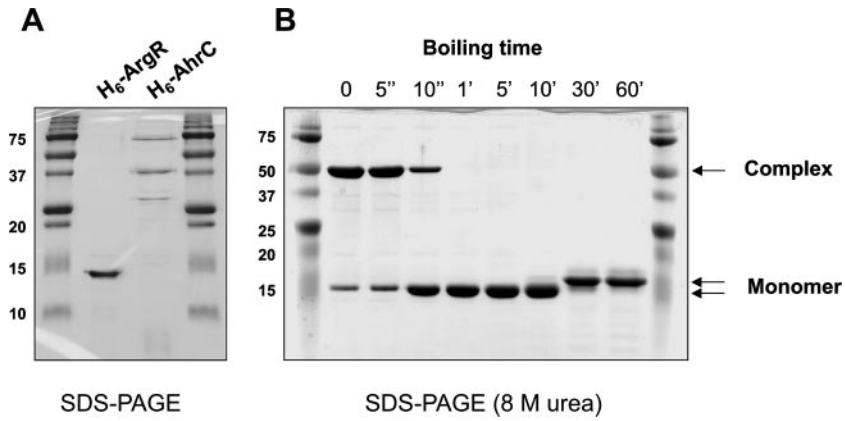


FIG. 1. **Purification of His_6-ArgR and His_6-AhrC.** *A*, SDS-PAGE of final pooled protein fractions after affinity fast protein liquid chromatography purification. The Precision Plus Protein Standard Dual Color marker (Bio-Rad) was used as reference. *B*, boiling and subsequent electrophoretic separation of purified His_6-AhrC in 8 M urea. Boiling time is indicated *above* each *lane*. The high molecular weight complexes and bands of monomeric proteins are indicated by *arrows*.

site of the P*nisA* expression vector pNG8048E, resulting in the plasmids pNG-HisArgR and pNG-HisAhrC. The expression constructs were made and maintained in NZ9000Δ*argRahrC* and overexpression of the His-tagged regulators, His_6-ArgR and His_6-AhrC, was induced by the addition of Nisaplin (as described above) to the cultures during the midexponential phase of growth in GM17. After induction for 2 h, 900 ml of cell culture were harvested, washed, and resuspended in 16 ml of column buffer (250 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris-HCl (pH 8.0), 10% glycerol, 1 mM β-mercaptoethanol). Cells were disrupted by shaking twice for 1 min at room temperature with glass beads (75–105 μm) in a Biospec Mini-BeadBeater-8 (Biospec). Samples were kept on ice between steps. Glass beads and cell debris were removed by two centrifugation steps (5 min at 14,000, 4 °C). Proteins were purified by affinity fast protein liquid chromatography; crude cell extracts were applied to Ni^{2+}-nitrilotriacetic acid Superflow resin (Qiagen GmbH, Hilden, Germany) and washed with column buffer until complete removal of bulk proteins, followed by ~10 volumes of wash buffer (column buffer plus 18.75 mM imidazole). Elution was done with elution buffer (column buffer plus 250 mM imidazole). Elution fractions and pooled fractions after removal of imidazole were analyzed for yield and purity by SDS-PAGE. Imidazole was removed from the eluate by dialysis, using dialysis membranes from Medicell International Ltd. (London, UK). Since dialysis of His_6-ArgR resulted in significant protein precipitation, imidazole was removed from His_6-ArgR samples using a PD-10 desalting column (Amersham Biosciences). Protein concentration was determined by the method of Bradford (34). In-gel samples of purified His_6-AhrC were analyzed by MALDI-TOF[1] (Analytical Biochemistry, Department of Pharmacy, University of Groningen, The Netherlands).

[1] The abbreviation used is: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

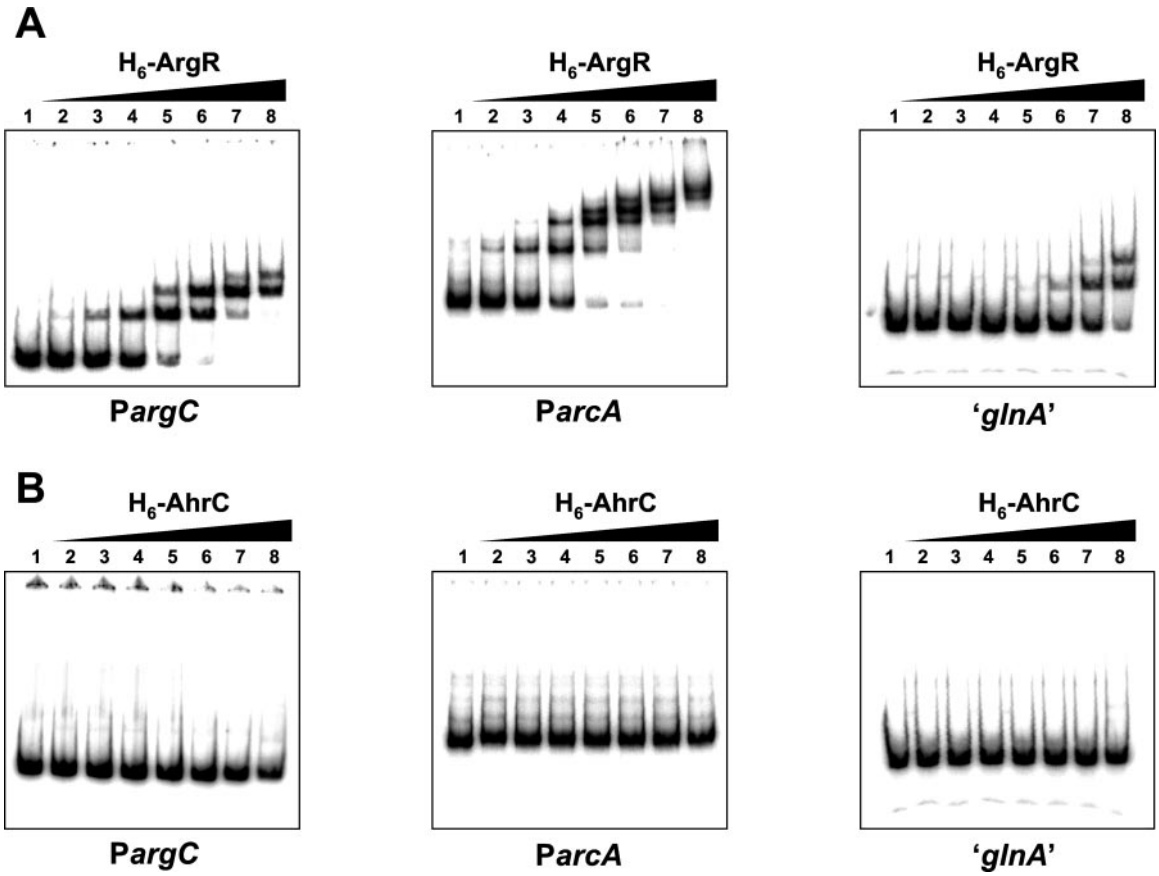


FIG. 2. **Analysis of binding of His_6-ArgR and His_6-AhrC to P*argC*, P*arcA*, and *glnA* by electrophoretic mobility shift assay.** The P*argC* fragment (141 bp), the P*arcA* fragment (282 bp), and the *glnA* fragment (134 bp) were obtained by PCR with primers specified in Table II. End-labeled probes were incubated with His_6-ArgR or His_6-AhrC, and retardation was investigated by electrophoresis on 6% polyacrylamide gels. *A*, His_6-ArgR was used in the following concentrations (in monomer equivalents). *Lanes 1*, no regulator. *Lanes 2–8*, 3.4×10^{-11}, 1.7×10^{-10}, 8.6×10^{-10}, 4.3×10^{-9}, 2.2×10^{-8}, 1.1×10^{-7}, and 5.4×10^{-7} M, respectively. *B*, His_6-ArhC was used in the following concentrations (in monomer equivalents). *Lanes 1*, no regulator. *Lanes 2–8*, 2.5×10^{-10}, 1.3×10^{-9}, 6.4×10^{-9}, 3.2×10^{-8}, 1.6×10^{-7}, 7.9×10^{-7}, and 4.0×10^{-6} M, respectively. All samples were preincubated in binding buffer containing 10 mM arginine.

Construction of Regulator Point Mutations—The *argR* and *ahrC* genes, amplified by PCR using the argR-NZ/argR-MAL2 and ahrC-NZ/ahrC-5 primer pairs, respectively, were blunt end-cloned into the SmaI restriction site of pUC19 (35). The proper constructs were picked up in *E. coli* XL1-Blue. Point mutations were introduced in *argR* and *ahrC* using the protocol of the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). Whole-plasmid PCR was performed using the native *Pfu* DNA polymerase (Stratagene) to make the following ArgR and AhrC mutations (primers used are shown in parentheses): ArgR-A126D (argR-MG(A126D)-1/argR-MG(A126D)-2); ArgR-D127G (argR-MG(D127G)-1/argR-MG(D127G)-2); AhrC-D124G (ahrC-MG-(D124G)-1/ahrC-MG(D124G)-2); AhrC-D126G (ahrC-MG(D126G)-1/ahrC-MG(D126G)-2) (see Table II). Mutations were verified by nucleotide sequencing, and mutated genes were subsequently cloned as RcaI/HindIII and RcaI/XbaI restriction fragments into the NcoI/HindIII and NcoI/XbaI sites of the P*nisA* expression vector pNG8048E, respectively, using *L. lactis* NZ9000 as the cloning host. The plasmid constructs were again verified by nucleotide sequencing and used to transform *L. lactis* strains NZΔ*ahrC* and NZΔ*argRahrC*.

Gel Retardation Assays—DNA binding of His_6-ArgR and His_6-AhrC was investigated by gel retardation (band shift) assays, essentially as described by Ebbole and Zalkin (36). Probes were amplified using *Pwo* DNA polymerase with corresponding primer pairs as follows: P*argC*, argC-2/argC-5; P*arcA*, arcA-1/arcA-7rev; "*glnA*," glnA-3/glnA-5; 1/1rev, arcA-1/arcA-1rev; 3/3rev, arcA-3/arcA-3rev; 4/4rev, arcA-4/arcA-4rev; 5/5rev, arcA-5/arcA-5rev; 6/6rev, arcA-6/arcA-6rev; 7/7rev, arcA-7/arcA-7rev; 10/10rev, arcA-10/arcA-10rev (see Table II). PCR products (~2 μg) were end-labeled with 30 μCi of [γ-^{32}P]ATP using polynucleotide kinase (Amersham Biosciences) for 2 h at 37 °C. Reactions were stopped by heating for 10 min at 70 °C, and labeled probes were purified with the Roche Applied Science PCR product purification kit. Binding reactions were performed in binding buffer (20 mM Tris-HCl (pH 8.0), 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 8.7% (v/v) glycerol, 25 μg/ml bovine serum albumin, and 50 μg/ml poly(dI-dC)) with 5000 cpm of labeled probe, in a final volume of 20 μl. Varying concentrations of His_6-ArgR, His_6-AhrC, and arginine were used as specified. Reactions were incubated for 30 min at 25 °C and analyzed by electrophoresis (~1 h at 90 V) in 6% polyacrylamide gels, using the Protean II Minigel System (Bio-Rad) with TBE as electrophoresis buffer. Gels were vacuum-dried and developed using a Cyclone Phosphor Screen Storage system (Packard Bioscience) and OptiQuant software version 3.0 for analysis (Packard Instrument Co). The intensity of single bands was measured using Quantity One software, version 4.1.0 (Bio-Rad), and the apparent equilibrium dissociation constants (K_d) were calculated as the concentration of regulator at which 50% of the free probe was shifted.

DNase I Footprinting Assays—His_6-ArgR and His_6-AhrC DNA binding sites were analyzed by DNase I footprinting (protection) assays, largely according to the protocol of the Sure Track Footprinting Kit (Amersham Biosciences). The P*argC* region was amplified with the argC-7 (forward) and argC-2 (reverse) primers, one of which was end-labeled (2 h at 37 °C) with [γ-^{32}P]ATP using T4 polynucleotide kinase (Amersham Biosciences) according to the manufacturer's instructions, before standard PCR with the respective unlabeled primers. The P*arcA* region was likewise amplified and labeled, using the arcA-1 (forward) and arcA-7rev (reverse) primers. Binding reactions were performed as described for the gel retardation assays (see above), except that the final volume was 40 μl, and 150,000 cpm of labeled DNA was used per lane. Concentrations of His_6-ArgR, His_6-AhrC, and arginine were as specified. DNase I (Amersham Biosciences) degradation and fragment separation by polyacrylamide gel electrophoresis (National Diagnostics) were performed as described previously (37). Detection was performed as described for the gel retardations (see above). Maxam-Gilbert sequencing reactions were made from the footprinting probes according to Sambrook *et al.* (29) and were run next to the footprinting lanes to determine the sizes of degradation fragments.

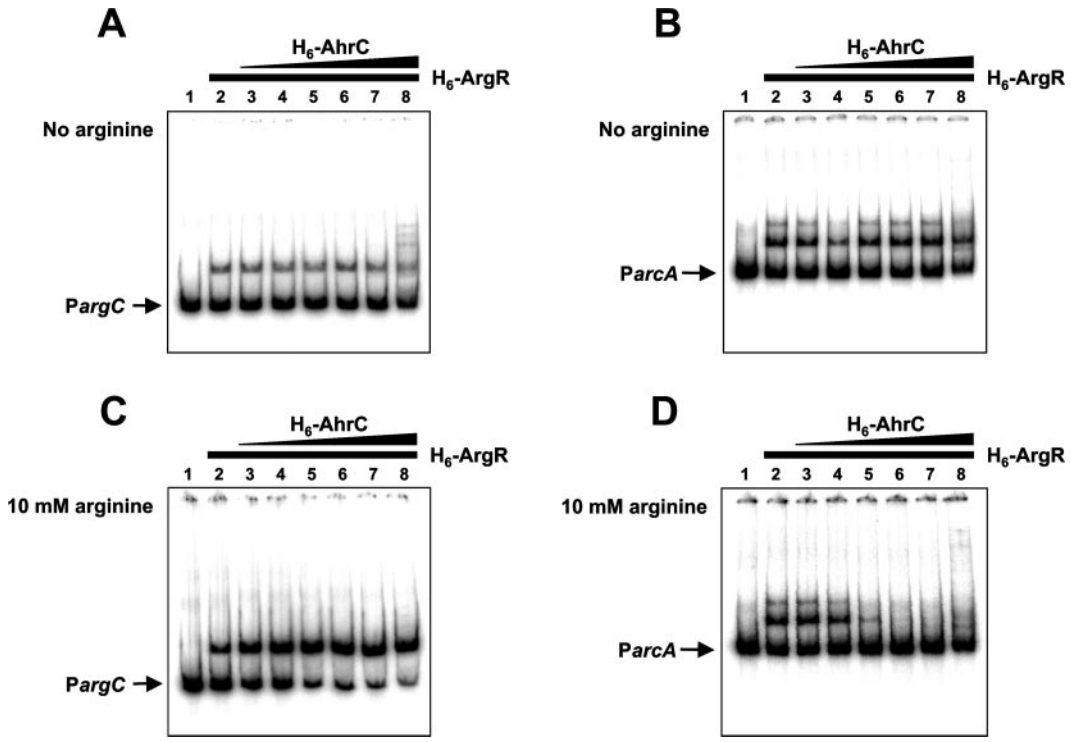


FIG. 3. **Analysis of arginine-dependent interaction between His_6-ArgR and His_6-AhrC by electrophoretic mobility shift assays.** End-labeled PCR probes for P*argC* and P*arcA* were used as described in the legend to Fig. 2. Probes were incubated with a fixed concentration of His_6-ArgR of 1.3×10^{-10} M (monomer equivalents) in *A* and *C*, and 6.7×10^{-10} M in *B* and *D*, in the absence (*A* and *B*) and presence (*C* and *D*) of 10 mM arginine. No regulator protein was present in *lanes 1*. His_6-AhrC was used in the following concentrations (monomer equivalents). *Lanes 2*, no His_6-AhrC; *lanes 3–8*, 1.3×10^{-9}, 6.4×10^{-9}, 3.2×10^{-8}, 1.6×10^{-7}, 7.9×10^{-7}, and 4.0×10^{-6} M, respectively. Nonshifted probes are indicated with *arrows*.

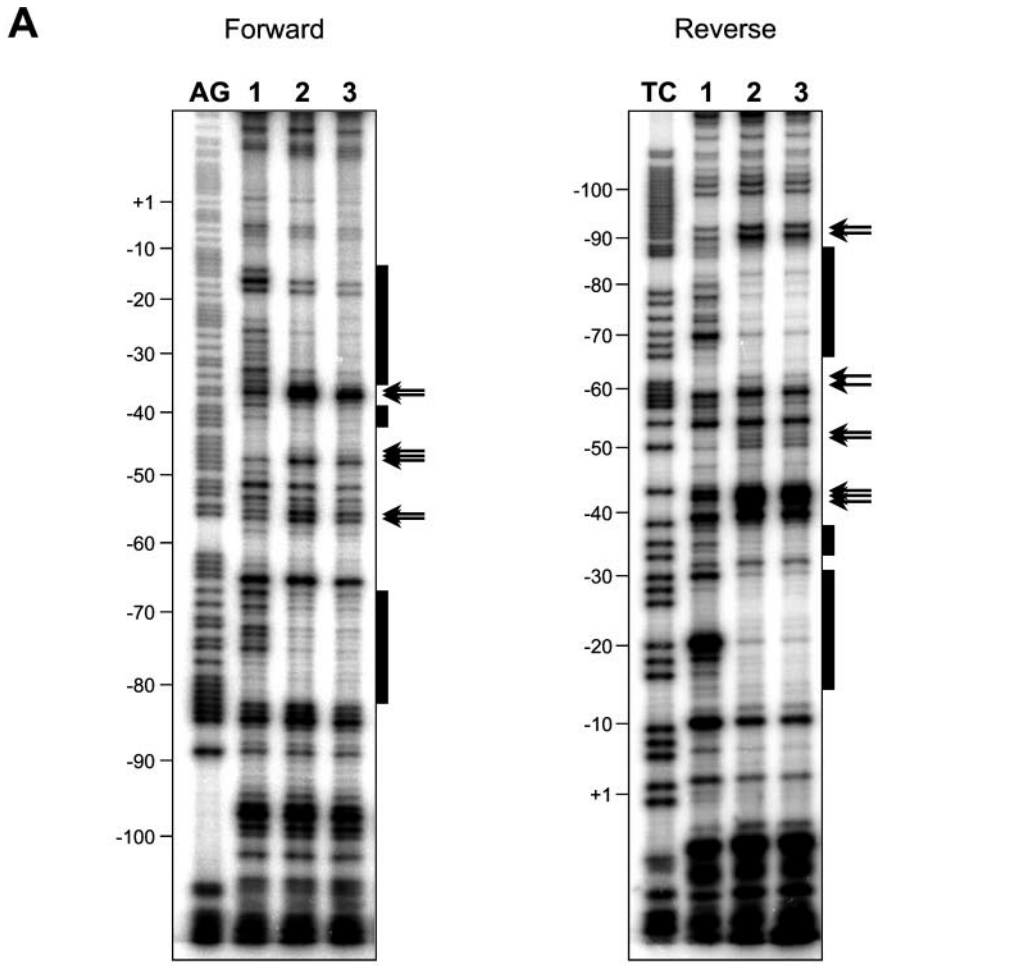
RNA Isolation and Primer Extension—RNA was isolated from cells grown to the midexponential phase of growth (A_{600} = 0.6–0.7) in CDM15 with 0.1 or 10 mM arginine. RNA isolation was carried out using macaloid to remove DNA and the High Pure RNA isolation kit (Roche Applied Science). RNA quantity was determined spectrophotometrically (29), and RNA quality was verified on an Agilent Bioanalyzer 2100 using RNA 6000 LabChips (Agilent Technologies Netherlands BV, Amstelveen, The Netherlands). Oligonucleotide arcA-px was end-labeled with [γ-^{33}P]ATP using T4 polynucleotide kinase (Amersham Biosciences) and purified with the QIAquick nucleotide removal kit (QIAgen GmbH, Hilden, Germany). The labeled oligonucleotide was used for synthesis of first strand cDNA with 5 μg of total RNA as template, using the SuperScriptIII reverse transcriptase (Invitrogen) by incubating for 10 min at 25 °C and 40 min at 42 °C. The enzyme was inactivated by heating at 70 °C for 15 min. Primer extension products were analyzed by electrophoresis (National Diagnostics) next to a Maxam-Gilbert sequencing ladder, made from an arcA-px/arcA-1 PCR product, using the [γ-^{33}P]ATP end-labeled arcA-px oligonucleotide, as described for footprinting assays (see above).

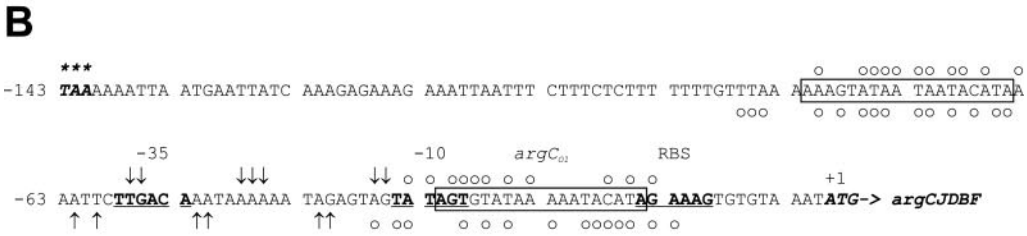
Citrulline Determination—Intracellular citrulline concentrations were determined in cell-free extracts of *L. lactis* strains harvested at the midexponential phase of growth in CDM15 with 10 mM arginine. Citrulline measurements were done essentially according to Archibald (38).

RESULTS

Isolation of His_6-ArgR and His_6-AhrC Reveals an Unusually Stable Multimeric Complex of AhrC—DNA fragments encoding N-terminally hexahistidine-tagged derivatives of the two arginine regulators ArgR and AhrC of *L. lactis* MG1363 were cloned behind the nisin-inducible *nisA* promoter in pNG8048E. The His-tagged regulators, His_6-ArgR and His_6-AhrC, were overproduced in *L. lactis* NZ9000Δ*argRahrC* to prevent co-purification with the wild-type regulator proteins and isolated to near purity as determined by SDS-PAGE (Fig. 1). His_6-ArgR appeared as a protein of ~15 kDa, which is well in agreement with the expected size of the regulator in monomeric form (Fig. 1*A*). During our studies, His_6-AhrC consistently appeared in several bands corresponding to high molecular weight proteins when investigated by SDS-PAGE (Fig. 1*A*). Expression of the wild-type AhrC protein in *L. lactis* as well as in *E. coli* BL21(DE3) also yielded high molecular bands in SDS-PAGE, in addition to a band expected for the monomeric form of the protein, despite sample boiling prior to electrophoresis and electrophoresis under denaturing conditions (data not shown). Thus, the stable His_6-AhrC complexes were not caused by the His tag. The ability of the His-tagged regulators to complement the *argR* and *ahrC* deletion mutants was examined by measuring intracellular citrulline. These studies showed that the His tags did not abolish regulator functionality (data not shown). To make sure that the high molecular weight bands observed during SDS-PAGE of His_6-AhrC were not caused by contaminating, co-purified proteins, proteins in these bands were proven to be identical to AhrC of *L. lactis* by MALDI-TOF analysis (data no shown). Furthermore, purified samples of His_6-AhrC were denatured by incubation and electrophoresis in 8 M urea (Fig. 1*B*). Samples were boiled for increasing periods of time until complete dissociation to the monomeric form was observed (Fig. 1*B*). Surprisingly, boiling of up to 30 s in 8 M urea was required for complete denaturation of the high molecular weight His_6-AhrC form, which indicates that this regulator forms unusually stable multimeric structures. Whether this is a result of overexpression or *in vitro* purification conditions remains to be determined.

Gel Retardation Experiments Reveal Differences between ArgR and AhrC—The functions of ArgR and AhrC were initially investigated by gel retardation experiments. Our earlier studies have shown that both regulators are involved in transcriptional repression of the arginine biosynthetic genes. A more complex mechanism, also requiring both regulators, is responsible for regulation of arginine catabolism (12). DNA fragments covering the biosynthetic *argC* and the catabolic *arcA* promoter regions were chosen as probes in gel retarda-





C

$argC_{01}$	AGTGTATAAAAATACATA
$argC_{02}$	AAAGTATAATAATACATA
$gltS_{01}$	ATTGTATAATTATACTTA
$gltS_{02}$	ATAAAATAAAATATGCAAT
$gltS_{03}$	AAAGTATAATAGTGCAGG
$argG_{01}$	AATGAATAAAATATGCTGT
$argG_{02}$	AACGCATATTTATACAAA
	----------
*Ll*_cons	AwwGwATAAWWATrCWww
*Ec*_cons	TnTGnATWWWWATnCAnA

FIG. 4. *A*, analysis of His_6-ArgR-His_6-AhrC binding to both strands of the 141-bp P*argC* fragment by DNase I footprinting. Regions of protection from nuclease attack are indicated by *black bars*, and sequence locations are indicated by *numbering* relative to the distance in bp from the *argC* translational start site. Hypersensitive sites are indicated by *horizontal arrows*. The *lanes* on each gel are as follows. *AG* and *TC*, Maxam-Gilbert

tion experiments. An intragenic *glnA* fragment was used as negative control. His_6-ArgR was able to shift all three probes (Fig. 2*A*). The apparent dissociation constants (K_d) for His_6-ArgR were calculated to be 1.4×10^{-9} M for P*argC*, 5.3×10^{-10} M for P*arcA*, and 1.3×10^{-7} M for the unspecific *glnA* probe. Besides the ~2.5-fold higher affinity of His_6-ArgR for P*arcA* than for P*argC*, more protein-DNA complexes were obtained with P*arcA* than with P*argC* (Fig. 2*A*), suggesting the presence of more operator regions in the former. Surprisingly, His_6-AhrC did not retard any of these probes (Fig. 2*B*) despite the fact that the protein contains a highly conserved N-terminal DNA binding domain. Importantly, cell-free extracts of *L. lactis* with overexpressed AhrC also failed to shift any of these probes (data not shown). Retardation experiments using either His_6-ArgR or His_6-AhrC were performed without arginine or in the presence of 10 mM arginine, but no difference was observed. The binding of His_6-ArgR to the supposedly unspecific *glnA* probe revealed that ArgR has an intrinsic DNA binding ability and allowed us to work at His_6-ArgR concentrations that were specific for the *argC* and *arcA* promoter fragments during the remainder of the study.

Interaction between ArgR and AhrC Is Necessary for Regulation in Response to Arginine—The specific binding of His_6-ArgR to the catabolic *arcA* promoter as well as to the biosynthetic *argC* promoter, the lack of His_6-AhrC-DNA interaction, and the knowledge that both regulators are required for arginine-dependent regulation, led us to perform gel retardation experiments in the presence of both proteins. Using concentrations of His_6-ArgR that only partially shifted the free probes, His_6-AhrC was added, with and without 10 mM arginine (Fig. 3). The addition of His_6-AhrC had no effect on His_6-ArgR-mediated band shifts in the absence of arginine (Fig. 3, *A* and *B*). However, in the presence of arginine, clear but opposite effects were observed for the two different promoter fragments. Whereas His_6-AhrC increased the affinity of His_6-ArgR for P*argC* (Fig. 3*C*), His_6-ArgR-mediated interaction with P*arcA* was completely lost (Fig. 3*D*).

His_6-ArgR and His_6-AhrC Interact with ARG Box-like Operators in the Biosynthetic argC Promoter Region—The binding of His_6-ArgR-His_6-AhrC to the *argC* promoter region was further investigated by DNase I footprinting. The concentration of His_6-AhrC was increased in the presence of a fixed, low amount of His_6-ArgR and 10 mM arginine. Two operator sites of 20–25 bp, here called $argC_{O1}$ and $argC_{O2}$, were protected in both strands of the *argC* promoter fragment in an His_6-AhrC-dependent manner (Fig. 4*A*). Visual inspection of the protected residues showed that the two sites have high similarity to classical ARG box operators known to be required for binding of ArgR-type regulators in several organisms (5′-TnTGnATwww-wATnCAnA-3′, where n represents any nucleotide, w is A or T, and capitalized residues are highly conserved) (Fig. 4, *B* and *C*). The two ARG boxes are separated by a 32-bp spacer region that contains hypersensitive residues on both strands, suggesting that bending of DNA takes place between the two sites as a result of His_6-ArgR-His_6-AhrC binding (Fig. 4). DNase I footprinting experiments using P*argC* and His_6-ArgR alone did not give clearly protected sites (data not shown), possibly because of the weak affinity of His_6-ArgR for P*argC*, compared with that of His_6-ArgR-His_6-AhrC.

His_6-ArgR Binds to Several ARG Box Half-sites in the arcA Promoter Region—In contrast to the *argC* promoter region, no consensus ARG box(es) could previously be identified in the promoter region of *arcA*. Since His_6-AhrC diminishes the binding of His_6-ArgR to P*arcA* (Fig. 3), footprinting of this promoter region was performed with His_6-ArgR alone. Although binding of His_6-ArgR was weak, protected regions could still be discerned in P*arcA* (Fig. 5*A*). Interestingly, inspection of the protected sites revealed a high similarity of these to ARG box half-sites of the sequence 5′-TGnATAWW-3′ (where n represents any nucleotide; W is A or T; and capital letters represent conserved residues) (Fig. 5, *B* and *C*). Some of these ARG-half sites (called ARC boxes below) are positioned immediately next to each other without spacing, whereas others are present as single boxes (Fig. 5*B*). Weakly hypersensitive sites were identified between the sites denoted as C_1C_2 and D_1D_2, shown in Fig. 5, located on the predicted P2 and P1 promoter regions, respectively (Fig. 5).

To confirm the protection assays, overlapping P*arcA* fragments of the same size (~100 bp) were used in gel retardation assays. All fragments (except the *arcA* and *glnA* intragenic controls) gave low molecular weight complexes, whereas fragments containing the central *B* and C_1C_2 regions additionally resulted in a high molecular weight complex (Fig. 6). However, the relative amounts of shifted *versus* free probes define a center of binding around the 5/5rev fragment (Fig. 6*C*). Although direct correlation of these results to those of the protection assays is not possible, a number of important conclusions can still be drawn. First, His_6-ArgR binds specifically to several sites spanning the P*arcA* region. Second, the double ARC box at location C_1C_2 (Fig. 6*C*) is not essential for His_6-ArgR binding, since fragments that only contain the ARC boxes *A* or *D* were still retarded (Fig. 6). Third, strong His_6-ArgR binding was centered around the ARC boxes *B* and *C*, which cover the putative P2 promoter.

The arcA P1 Promoter Is Regulated in Response to Arginine—The *arcA* promoter region contains two core promoter sequences, suggesting that transcription of the *arc* operon genes might initiate and/or be regulated at two different sites. To answer this question, primer extension analysis was performed using total RNA isolated from the wild type strain *L. lactis* MG1363, the arginine regulator single mutants MGΔ*argR* and MGΔ*ahrC*, and the double mutant MGΔ*argRahrC*, grown in high (10 mM) or low (0.1 mM) concentrations of arginine. A primer annealing ~100 bp downstream from the −10 region of *arcA* P1, was used in the reverse transcription reactions. Only very weak bands were observed in the 33-bp space between P2 and P1, suggesting that P2 has no or only low activity under the conditions applied. In contrast, a strong band appeared at a T residue 6 bp downstream of P1, indicating that P1 most likely is the main *arc* promoter (Fig. 7). Additionally, the primer extensions showed that transcription from *arcA* P1 is strongly regulated by the availability of arginine in the wild type strain (Fig. 7). In the *ahrC* deletion strain, no expression was seen, and in the *argR* single mutant and the *argR ahrC* double mutant, high expression was observed irrespective of the arginine concentration in the growth medium (Fig. 7).

AhrC(Asp^{124}) Is Important for Arginine-dependent Activation of the Arginine Catabolic Operon—The three-dimensional

sequence ladder; *lane 1*, 0 M; *lane 2*, 6.6×10^{-8} M; *lane 3*, 6.6×10^{-7} M His_6-AhrC, respectively (in monomer equivalents). Each *numbered lane* additionally contains 1.3×10^{-8} M His_6-ArgR (monomer equivalents), and all samples were preincubated in the presence of 10 mM arginine. *B*, sequence of the *argC* promoter region. The *numbers* show distance in bp to the *argC* translational start site (in *italic type*); the −35 and −10 motifs of P*argC* are in boldface type, and the ribosomal binding site is *underlined*. The *open circles* indicate residues protected in footprints on the forward strand (*above* the sequence) and reverse strand (*below* the sequence). Hypersensitive residues are indicated by *vertical arrows*. *C*, alignment of the *L. lactis* ARG box operators and resulting consensus sequence: $argC_{O1}$ and $argC_{O2}$ are from this study; $argG_{O1}$, $argG_{O2}$, $gltS_{O1}$, and $gltS_{O2}$ are predicted from promoter sequences (12). The *E. coli* ARG box consensus is according to Maas (21). The *convergent arrows* indicate ARG box dyad symmetry.

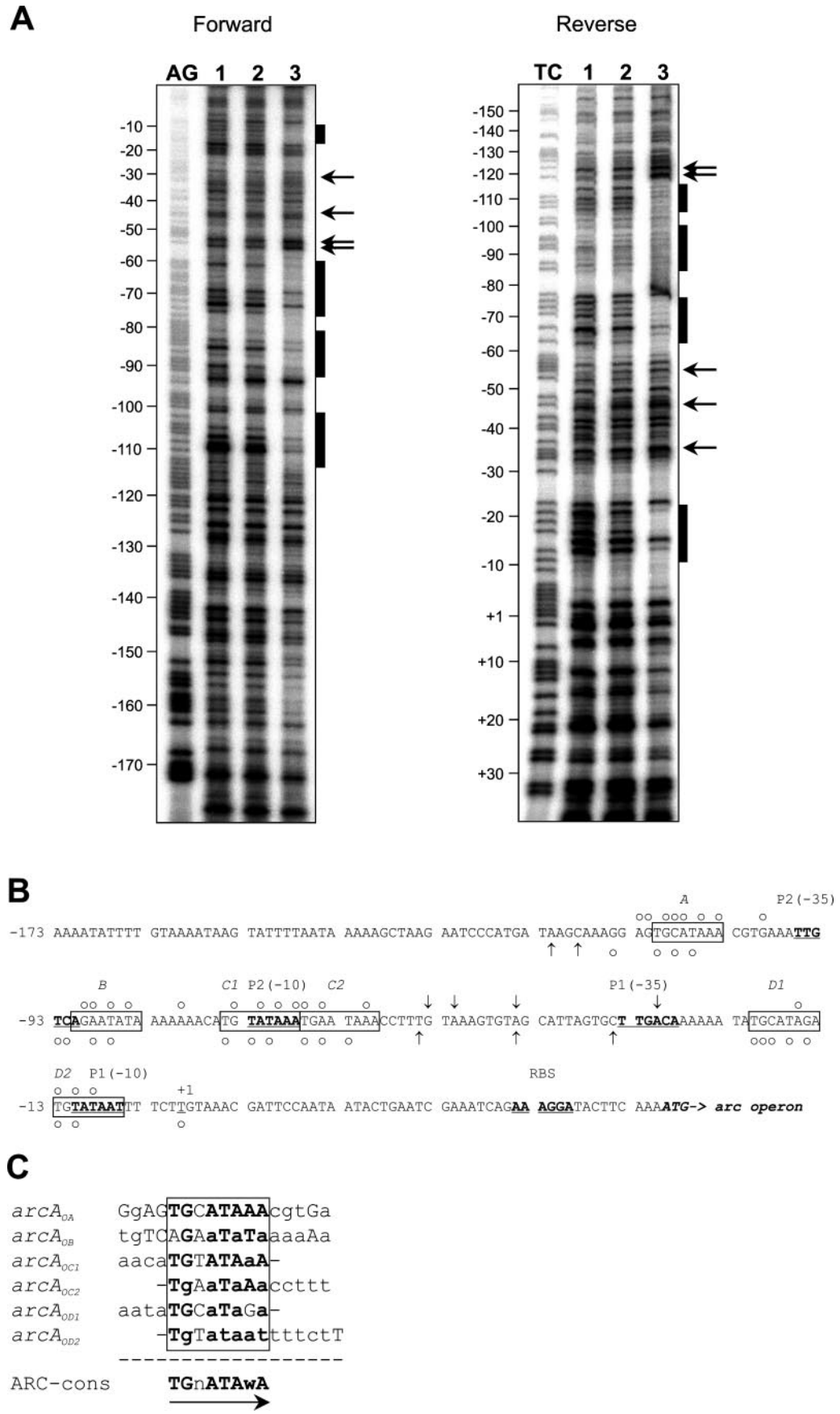


FIG. 5. *A*, analysis of binding of His_6-ArgR on both strands of the 282-bp P*arcA* fragment by DNase I footprinting. Regions protected from nuclease attack are indicated by *black bars*, and sequence locations are indicated by numbering in bp relative to the *arcA* transcriptional start site. Hypersensitive sites are indicated by the *horizontal arrows*. The *lanes* on each gel are as follows. *AG* and *TC*, Maxam-Gilbert sequence ladder. *Lane 1*, 0 M; *lane 2*, 9.0×10^{-9} M; *lane 3*, 9.0×10^{-8} M His_6-ArgR, respectively (in monomer equivalents). *B*, sequence of the *arcA* promoter region. The *numbers* show distance in bp to the *arcA* transcriptional start site (+1); the −35 and −10 motifs of *arcA* P1 and P2 are in *boldface type*,

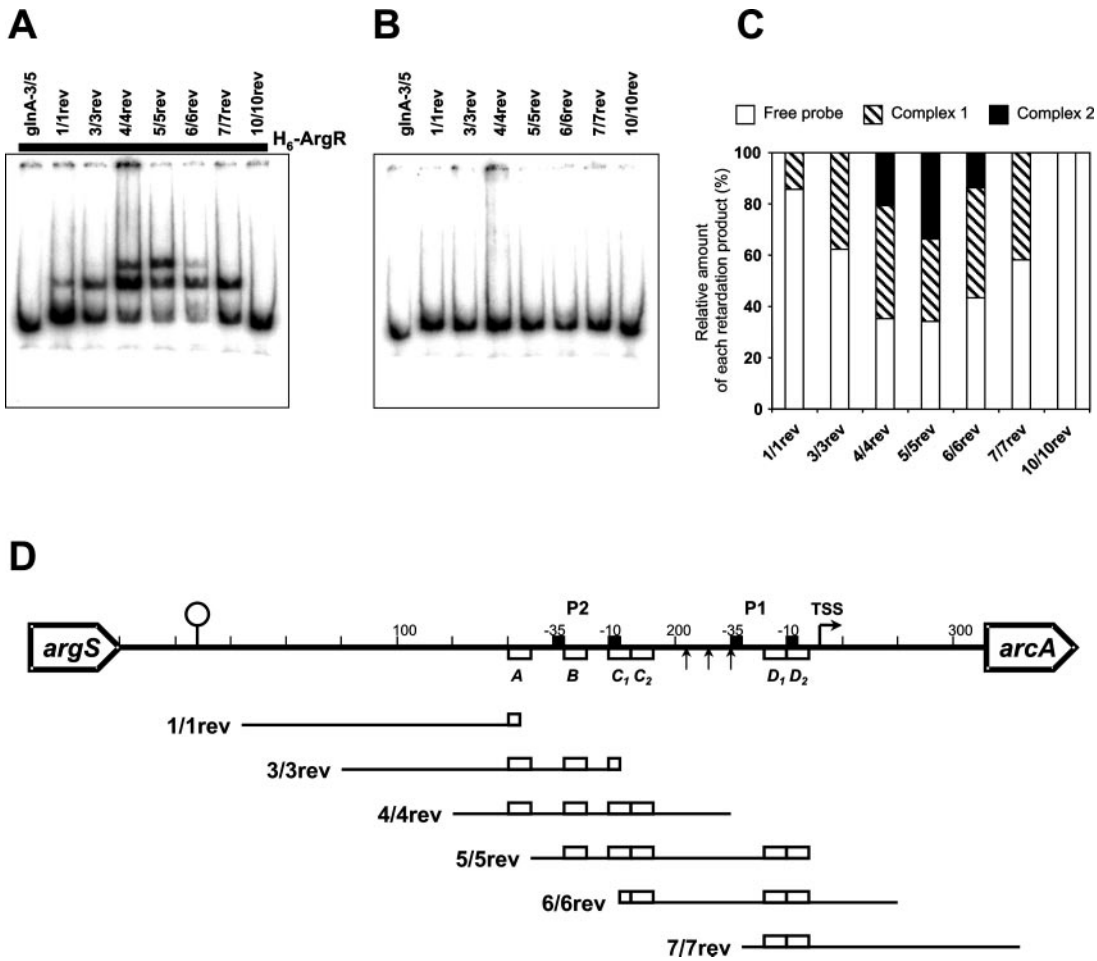


FIG. 6. **Gel retardation analysis of His_6-ArgR interaction to P*arcA* fragments.** Each sample contained the same concentration of end-labeled PCR probe and 6.7×10^{-9} M His_6-ArgR (in monomeric equivalents) (*A*) or no regulator (*B*). The *glnA* intragenic glnA-3/5 probe (134 bp) was used as negative control. P*arcA* probes were 1/1rev (116 bp), 3/3rev (116 bp), 4/4rev (116 bp), 5/5rev (116 bp), 6/6rev (116 bp), and 7/7rev (116 bp). 10/10rev is an *arcA*-intragenic probe (108 bp). *C*, relative amounts (in percentages) of free probe (*white*), low molecular weight complex (*striped*), and high molecular weight complex (*black*) in P*arcA* shifts (*lanes 2–8* in *A*). *D*, schematic representation of the *argS-arcA* intergenic region. The *black squares* indicate the −10 and −35 motifs of the P1 and P2 core promoter regions, and the transcription start site (*TSS*) is shown with a *bent arrow*. A terminator structure is indicated by a *lollipop*. The *white boxes below* the DNA line represent ARC operator sites, labeled *A–D*. PCR fragments used in the gel retardation assays in *A*, 1/1rev to 7/7rev, are indicated as *horizontal lines*.

structures of ArgR-type regulators from *E. coli*, *B. stearothermophilus*, and *B. subtilis* have shown that arginine bound to the proteins interacts with two conserved aspartate residues in the C-terminal sensing domain. However, the situation is different in *L. lactis* and other low-G + C Gram-positive organisms (Fig. 8*A*). ArgR of *L. lactis* has only one of the two Asp residues, whereas AhrC has three (12) (Fig. 8). In order to evaluate the importance of these Asp residues in the regulators in *L. lactis*, two mutations were introduced in each regulator (see Fig. 8*B*). The function of the mutated regulators was determined by expression in *ahrC* and *argR ahrC* mutants of *L. lactis* NZ9000. The intracellular concentration of citrulline in the strain was determined as a measure of arginine degradation via the *arc* operon-encoded arginine deiminase (ADI) pathway. ArgR(D127G) and ArgR(A126D) behaved like wild type ArgR. Thus, the conserved ArgR(D127) is not important for arginine sensing in *L. lactis*, and the introduction of an Asp residue at ArgR(Ala^{126}) could not complement the AhrC deletion. Considering that ArgR(Asp^{127}) and AhrC(Asp^{126}) of *L. lactis* are conserved in all aligned regulators (Fig. 8*A*), it was surprising that also AhrC(D126G) activity was almost that of the wild type AhrC. However, the "extra" Asp^{124} of AhrC is of major importance for activity, since AhrC(D124G) resulted in a drastic reduction of citrulline production via the ADI pathway (Fig. 8*B*).

DISCUSSION

The work presented in this paper was aimed at clarifying the specific functions of the two arginine regulators ArgR and AhrC in arginine metabolism and regulation of *L. lactis*. We approached the question mainly by gel retardation and footprinting analysis. Eventually, the obtained results, to be discussed below, have led us to propose the regulatory model shown in Fig. 9.

Since putative ARG box operators could be predicted in the *argC* promoter region (12), His_6-ArgR binding to this promoter fragment was expected. However, His_6-ArgR showed even higher affinity (~2.5-fold) for the *arcA* promoter, which lacks consensus ARG box sequences, than for the *argC* promoter. The observation that the addition of arginine had no effect on His_6-

and the ribosomal binding site is *underlined*. The *open circles* indicate residues protected in footprints on the forward strand (*above* the sequence) and reverse strand (*below* the sequence). Hypersensitive residues are indicated by *vertical arrows*. *C*, alignment of the *L. lactis* ARC boxes and resulting consensus sequence. The *arrow* underneath the alignment refers to one-half of an ARG box (compare with Fig. 4*C*).

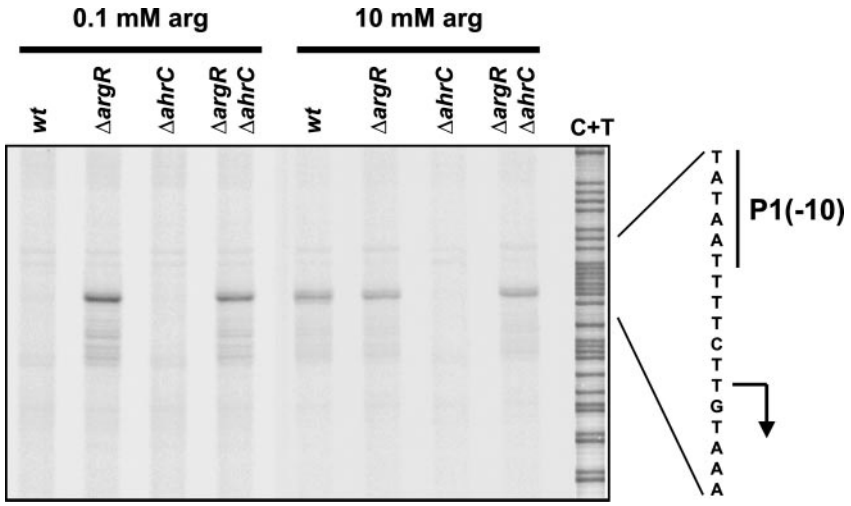


FIG. 7. **Primer extension of the *arcA* promoter.** Primer extension reactions were performed with primer arcA-px on total RNA isolated from MG1363 (wild type), MGΔ*argR*, MGΔ*ahrC*, and MGΔ*argRahrC*, harvested at midexponential phase of growth in CDM15, with 0.1 and 10 mM arginine. *C* + *T*, Maxam-Gilbert sequencing ladder made from PCR product of primer arcA-px (labeled) and arcA-1 (unlabeled). Part of the *arcA* promoter region, including the −10 region of promoter P1, indicating the exact transcriptional start site is shown on the *right*.

A

Eco_ArgR	^{126}AG**DD**TI^{131}
Bst_ArgR	^{123}CG**DD**TC^{128}
Bsu_AhrC	^{123}CG**DD**TI^{128}
Tne_ArgR	^{126}AG**DD**TI^{131}
Tma_ArgR	^{126}AG**DD**TI^{131}
Lla_ArgR	^{124}AGA**D**TL^{129}
Lla_AhrC	^{123}I**DDD**SA^{128}
Lpl_ArgR1	^{123}AGH**D**TI^{128}
Lpl_ArgR2	^{122}LG**DD**AH^{127}
Efa_ArgR1	^{126}AGF**D**TV^{131}
Efa_ArgR2	^{129}AG**DD**TI^{134}
Efa_ArgR3	^{124}AGA**D**TI^{129}
Efa_ArgR4	^{122}N**DDD**SV^{127}

B

Plasmid/construct	Mutation	Citrulline determination in strain, NZΔ*ahrC*	Citrulline determination in strain, NZΔ*argRahrC*
pNG8048E (empty)	(no reg.)	1.4	21.8
pNG-ArgR (wt)	-AGADTL-	1.2	6.8
pNG-ArgR(A126D)	---D----	1.3	7.9
pNG-ArgR(D127G)	----G---	1.3	7.7
pNG-AhrC (wt)	-IDDDSA-	10.3	19.8
pNG-AhrC(D124G)	--G-----	3.2	15.2
pNG-AhrC(D126G)	----G---	7.8	14.9

FIG. 8. **Study of putatively arginine-binding residues in ArgR type regulators.** *A*, alignment of double-aspartate arginine binding motif in C-terminal domains of ArgR regulators in *E. coli* (*Ec_ArgR*), *B. subtilis* (*Bsu_AhrC*), *B. stearothermophilus* (*Bst_ArgR*), *T. neapolitana* (*Tne_ArgR*), *T. maritima* (*Tma_ArgR*), *L. lactis* (*Ll_ArgR* and *Ll_AhrC*), *L. plantarum* (*Lpl_ArgR1* and *Lpl_ArgR2*), *E. faecalis* (*Efa_ArgR1*, *Efa_ArgR2*, *Efa_ArgR3*, and *Efa_ArgR4*). The *superscript numbers* refer to the amino acid residues of the respective regulator sequence. *B*, citrulline determination of cell-free extracts from cultures harvested at midexponential phase of growth in CDM15, 10 mM arginine. Data are from one representative experiment of several.

ArgR binding suggested that ArgR does not carry out arginine regulation alone or that arginine is not the actual ArgR effector molecule. Equally surprising was the lack of probe-DNA binding by His_6-AhrC although the protein contains an N-terminal H-T-H DNA-binding domain that is highly conserved among ArgR-type regulators (12). The observation that deletion of *ahrC* results in derepression of arginine biosynthesis led to the initial conclusion that AhrC binds to ARG operators preceding the arginine biosynthetic genes. The stable His_6-AhrC complex could explain these inconsistencies. However, the ability of His_6-AhrC to complement an *ahrC* mutation, the fact that a high molecular weight complex was also observed when overexpressing wild type AhrC in *E. coli* as well as in *L. lactis*, and the failure of overexpressed wild type AhrC to mediate DNA binding as well motivated us to proceed with His_6-AhrC in these studies.

The arginine-independent DNA binding of His_6-ArgR and the lack of binding by His_6-AhrC, together with the knowledge that both regulators are required for arginine regulation (12), prompted us to perform gel retardation experiments, using both His_6-ArgR and His_6-AhrC. Indeed, arginine-dependent interaction with DNA fragments containing ARG or ARC boxes only took place in the presence of both regulators. His_6-AhrC increased the His_6-ArgR (or His_6-ArgR-His_6-AhrC) affinity for P*argC* considerably but decreased the His_6-ArgR affinity for P*arcA*. A peculiarity, however, was seen in the shifts of the *argC* promoter fragment. Since His_6-ArgR already forms a complex with the P*argC* probe, the increased shift correlating

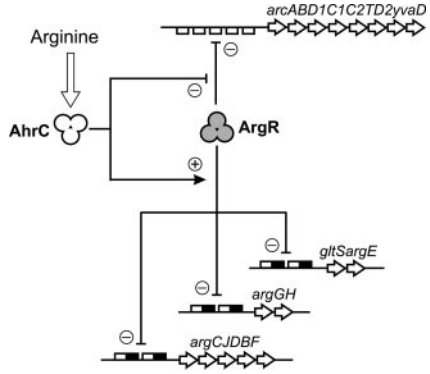


FIG. 9. **Schematic representation of regulatory mechanism employed by ArgR and AhrC in *L. lactis*, based on the results presented in this and previous studies.** The *plus signs* indicate positive regulatory effects, and *minus signs* indicate negative/inhibitory regulatory effects. ARC operator sites are shown as *white boxes*, and ARG operator sites are shown as *black and white boxes*, in the promoter regions of the arginine biosynthetic *argCJDBF*, *gltSargE*, and *argGH* operons and the arginine catabolic *arcABD1C1C2TD2yvaD* operon.

with the increase in the concentration of His_6-AhrC could be expected to result in the formation of one or more additional retardation complexes. This was not the case, since even under conditions where an almost complete shift (Fig. 3*C*, *lane 8*) of the P*argC* probe was seen, only a single retardation band was observed. One explanation for this result could be that AhrC transmits the arginine signal to ArgR, without actually binding

to ArgR and/or DNA. Alternatively, ArgR and AhrC complexes could be able to exchange subunits. Since all ArgR-type regulators characterized in detail so far have trimeric or hexameric quarternary structure (as dimers of trimers (13–16)), it is possible that each regulator is able to form homogenic dimers of trimers and, in the presence of arginine, form a heterogenic structure (*e.g.* consisting of an ArgR trimer bound to a trimer of AhrC). Examination of *B. stearothermophilus* ArgR has shown that binding of arginine in the trimer-trimer interface results in a rotation of one trimer relative to the other, which is proposed to increase the specificity for interaction with ARG box operators (15). It is tempting to speculate that a putatively hexameric ArgR of *L. lactis* has affinity for ARC operators in the absence of arginine but that the interaction with arginine and AhrC results in increased affinity and specificity for ARG operators concomitant with a decrease in the affinity for ARC operators. Along the same line of reasoning, an interaction between the two arginine regulators, ArgR1 and ArgR2 of *L. plantarum*, was proposed in order to explain the observation that introduction of point mutations in any one of the two DNA binding domains resulted in complete derepression of arginine biosynthesis (11).

The operator sites in the *argC* promoter region are highly similar to the 18-bp ARG boxes of *E. coli* (21). The presence of identical motifs in the promoter regions of the two other arginine biosynthetic operons, *gltSargE* and *argGH* (12), is in good agreement with this result and suggests that a similar mechanism of transcriptional regulation takes place at these promoters. By combining the ARG boxes of all three biosynthetic promoters, we were able to construct an ARG box consensus sequence for *L. lactis* (Fig. 4*C*). Considering the increased affinity of His_6-ArgR-His_6-AhrC for the ARG operators compared with that of His_6-ArgR alone, plus the conserved DNA binding domain of *L. lactis* AhrC, it is most likely that His_6-AhrC takes part in ARG box binding. Since ArgR is able to bind ARG box half-sites, one half of an ARG box might be occupied by ArgR, and the other half might be occupied by AhrC. This would also explain why the operators of the biosynthetic promoters and the catabolic promoter are different, namely to achieve differential regulation. The ARG boxes of *L. lactis* differ from those of most other systems by the presence of a large interoperator spacer region. Such spacer regions are generally 2–3 bp in *E. coli*, *B. stearothermophilus*, *B. subtilis*, and *Thermotoga neapolitana* (8, 21, 25), compared with 32 bp for the P*argC* operators and possibly 75 and 10 bp for the P*gltS* and P*argG* operators, respectively (with ARG box lengths of 18 bp). No clear difference in affinity of the regulators for $argC_{O1}$ and $argC_{O2}$ was apparent, and the presence of hypersensitive residues in the DNA footprint of the P*argC* region between the two operators suggests that DNA bending takes place. Bending could be the result of interaction between two regulators occupying the two sites or of looping of promoter DNA, leading to interaction of the DNA with two different DNA-binding regions of one regulator, as suggested for $argC_{O1}$ and $argC_{O2}$ of *B. subtilis* (4). Investigation of single box affinities will be required for elucidating the exact mechanism.

ArgR interacts with multiple operator sites (here called ARC sites), which are highly similar to ARG box half-sites and are present at various (about six) portions of the arginine catabolic *arcA* promoter region (Figs. 5 and 6). Interestingly, footprinting shows that ArgR protects single as well as double ARC boxes, and electrophoretic mobility shift assays using P*arcA* subclones suggest that ArgR-mediated regulation is centered around the C_1C_2 double ARC box (Fig. 6). Except for the D_1D_2 double box, all ARC boxes are located upstream of *arcA* P1, with the putative P2 core promoter sequence covered by as many as three boxes. Nevertheless, arginine-dependent transcriptional regulation appears to initiate at the *arc* operon proximal promoter *arcA* P1. An earlier P*arcA* deletion analysis using a low copy plasmid-encoded *lacZ* expression system revealed that expression of the *arcA* P1 minimal promoter was independent of arginine (12). By including the *arcA* P1 upstream region, corresponding to the 5′-ends of P*arcA* fragments 5/5rev and 6/6rev (Fig. 6*C*), arginine-dependent regulation was restored (12). The lack of regulation of *arcA* P1 lacking the upstream region, despite clear His_6-ArgR binding, can be explained in two ways; the low copy plasmid system may lead to insufficient *in vivo* levels of ArgR to repress the promoter, or, alternatively, interaction between regulator subunits binding to the D_1D_2 sites and the upstream *A*, *B*, and C_1C_2 sites might be required for efficient *arcA* P1 regulation. Expression and regulation of *arcA* P2 cannot be unequivocally excluded, but under the conditions applied, P2 does not seem to be regulated in response to arginine. It is noteworthy that the biosynthetic ARG boxes are composed of converging ARC boxes, explaining why His_6-ArgR (without His_6-AhrC) is able to shift the ARG box-containing fragments as well as those containing only ARC boxes. The necessity of ArgR binding to the A and B operator sites of P*arcA* is unclear but may be a drafting mechanism to attract ArgR molecules to the catabolic promoter.

Mutation of the double Asp residues in the C-terminal domain of ArgR*Ec* has been shown to be detrimental for arginine sensing (18, 19), and structural studies have suggested that these residues interact directly with arginine in the interface between the two ArgR trimers (13, 15, 16). Whereas double-Asp residues are conserved in ArgR regulators in organisms with a single ArgR regulator, large deviations in this region are observed in organisms with multiple ArgR-type regulators (Fig. 8*A*). Surprisingly, the fully conserved Asp^{129} (ArgR*Ec* numbering), was not essential for the functioning of ArgR*Ll* and AhrC*Ll*. Moreover, since ArgR(A126D) was unable to replace the function of AhrC*Ll*, these two residues are apparently not involved in arginine sensing in ArgR*Ll*. The additional Asp^{124} residue in AhrC, which is also present in ArgR4 of *E. faecalis*, was found to be of major importance for AhrC*Ll* functioning. Possibly, this Asp residue of AhrC*Ll* is able to complement the missing Asp residue of ArgR*Ll*. It is tempting to speculate that AhrC*Ll* and ArgR4*Efa* are responsible for arginine sensing, whereas the task of ArgR*Ll* and (at least one of) the other ArgR regulators of *E. faecalis* is DNA binding.

Based on the results presented here, we propose a model describing the functions of ArgR and AhrC in arginine-mediated transcriptional regulation in *L. lactis* (Fig. 9). In the absence of arginine, the higher affinity of ArgR for P*arcA* than for P*argC*, possibly due to the additional ARC sites in the former, suggests that ArgR mainly occupies the *arcABD1C1C2TD2yvaD* promoter, preventing arginine degradation via the ADI pathway. At the same time, this leaves expression of the arginine biosynthetic *argCJBDF*, *gltSargE*, and *argGH* operons unrepressed, allowing for *de novo* arginine production. The addition of arginine leads to association of ArgR and AhrC in a complex with high affinity for the ARG box operators. ArgR is shifted from the *arcA* promoter to the ArgR-AhrC complex, which represses expression of the arginine biosynthetic genes. Accordingly, the arginine catabolic *arc* operon is now derepressed, allowing for utilization of the arginine as a nitrogen and energy source via the ADI arginine degradation pathway (Fig. 9). With ArgR acting as the main transcriptional repressor, AhrC appears to have the unusual dual function of co-repressor and anti-repressor.

Despite the high conservation between ArgR-type regulators of different bacterial species, we show that the mechanisms by

which these proteins function are not conserved. This study extends our understanding of transcriptional regulation of arginine metabolism in organisms harboring more than one ArgR-type regulator, but intriguing questions remain to be answered. The subunit multimerization and overall structure of *L. lactis* ArgR and AhrC proteins is of major interest. Performing band shift or gel filtration experiments, using one wild-type regulator in combination with a functional fusion construct of the other, would verify the hypothesis of arginine-dependent interaction between ArgR and AhrC. Additionally, the multiple regulator system of *L. lactis* proposes that regulatory targets might exist in addition to the genes of the arginine metabolic pathways. Finally, the results presented here pose the question of why such a complex regulatory mechanism is operating in *L. lactis*, a renowned model organism because of its metabolic simplicity and low number of gene paralogues (39, 40).

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